Comprehensive Drug Analysis in Hair Samples: Extraction & UHPLC-MS/MS Quantification

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INTRODUCTION

The use of hair as a matrix for forensic toxicology continues to increase in popularity. As a specimen, hair offers several benefits. Sample collection is simple and easily supervised, and once collected, hair can be easily transported and stored at room temperature prior to analysis. Hair also provides an extended window of detection for drug exposure, enabling detection months and even years after use. This makes analysis of illicit drugs and pharmaceuticals in hair useful for situations where other samples may not be appropriate or available for analysis such as determining drug exposure in post-mortem toxicology, drug facilitated sexual assault (DFSA), or for other forensic testing in which long-term monitoring is desired.

The objective of this work was to develop, optimize and validate a method for the extraction and quantification of a comprehensive panel of drugs in hair to satisfy the confirmation cut-off values recommended by the society of Hair Testing (SoHT)[1]. This was accomplished by optimizing pulverization, incubation conditions, the analytical workflow and solid phase extraction (SPE). The sample preparation protocol resulted in consistent recoveries and well-controlled matrix effects. The resulting method was linear, accurate and precise for all target compounds and easily met the SoHT cut-off criteria for all target analytes.

METHODS

Chemicals: Certified reference materials and internal standards were from Millipore Sigma and Cayman Chemical.

Reagents: Reference standards were used to prepare working multi-analyte calibrator and QC solutions in methanol. External quality control samples were acquired from Comedical (Italy) and consisted of authentic hair with drugs incorporated into the keratin matrix with assigned values.

Sample preparation: Samples were decontaminated by sequential washing with aqueous buffer and solvents. Bulk hair samples were pulverized using a Precellys Tissue Homogenizer and 2 mL CKMix Lysing Kits (Bertin Technologies, Montigny-le-Bretonneux, FR) for 6 x 6400 rpm for 40 seconds each. Figure 1. shows a schematic of the pretreatment workflow. Samples were then extracted using Waters Oasis™ MCX 30 mg Plates. Figure 2 summarizes the SPE procedure

UHPLC-MS/MS analysis: A Waters ACQUITY™ UPLC™ I-Class (FTN) System was interfaced with a Xevo™ TQ Absolute Tandem Mass Spectrometer detector. Chromatography was performed using a Waters UPLC BEH™ C18 Column (1.7 µm, 2.1 x 100 mm) with a column temp. of 40°C. Mobile phase A (MPA) was 0.1% formic acid in water and mobile phase B (MPB) was 0.1% formic acid in LC-MS grade acetonitrile; flow rate was 0.6 mL/min. The LC gradient started at 2% B rising linearly to 67% MPB by 3.33 mins, then 90% B by 3.5 minutes before returning to 2% B from 3.6 to 4.0 mins. Injection volume was 2 µL. Mass spectrometer conditions were: source temperature 150°C, capillary voltage 1.0 kV desolvation gas (at 1000 L/h, 500°C) and cone gas (at 10 L/h). Data was processed with MassLynx™ Software and QUAN Review Application in the waters_connect™ Platform.

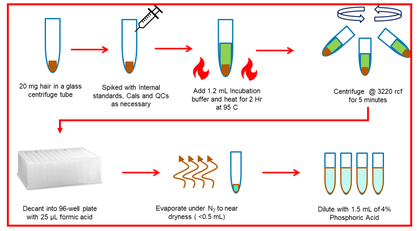


Figure 1. Graphical schematic of the pretreatment workflow

Figure 2. Solid Phase Extraction procedure

Analyte	RT	R^2
Morphine	0.89	0.998
Oxymorphone	0.94	0.999
Hydromorphone	1.02	0.999
Dihydrocodeine	1.20	0.997
Naloxone	1.2	0.998
Codeine	1.23	0.999
Noroxycodone	1.29	0.997
Amphetamine	1.31	0.995
Naltrexone	1.26	0.994
Oxycodone	1.27	0.999
6-acetyl morphine	1.28	0.996
MDA	1.29	0.995
Metadesnitazine	1.36	0.998
Hydrocodone	1.33	0.997
O-desmethyl Tramadol	1.32	0.999
Methamphetamine	1.35	0.998
MDMA	1.36	0.997
Phentermine	1.41	0.996
MDEA	1.47	0.999
Ritalinic acid	1.48	0.998
Norfentanyl	1.53	0.999
Benzoylecgonine	1.52	1.000
7-aminoclonazepam	1.71	0.999
Tramadol	1.67	0.999
N-desmethyltramadol	1.68	0.999
Methylphenidate	1.69	0.999
7-aminoflunitrazepam	1.71	0.999
Cocaine	1.79	1.000
Normeperidine	1.80	0.999

Analyte	RT	R^2
Meperidine	1.82	0.999
Norbuprenorphine	1.89	0.996
Chloriazepoxide	1.92	0.998
Trazodone	1.96	1.000
Cocaethylene	1.99	0.999
Phencyclidine	2.06	0.999
N-Pyrrolidino Etonitazine	2.17	0.994
Fentanyl	2.12	0.996
α-Hydroxymidazolam	2.12	0.996
Midazolam	2.15	0.998
Etonitazine	2.21	0.996
Flurazepam	2.20	0.999
Buprenorphine	2.24	0.996
EDDP	2.34	0.999
Methadone	2.55	0.996
α-Hydroxyalprazolam	2.55	0.998
α-Hydroxytriazolam	2.55	0.999
Nitrazepam	2.56	0.999
Oxazepam	2.63	0.999
Lorazepam	2.70	0.998
Clonazepam	2.69	0.999
Alprazolam	2.72	0.999
2-hydroxy-ethylflurazepam	2.72	0.999
Nordiazepam	2.73	0.999
Triazolam	2.77	0.998
Desalkylflurazepam	2.82	0.999
Flunitrazepam	2.83	0.998
Temazepam	2.91	0.998
Diazepam	3.10	0.999

Table 1. Target analytes, retention times and R^2 values from calibration curves

RESULTS

Recovery and Matrix Effects

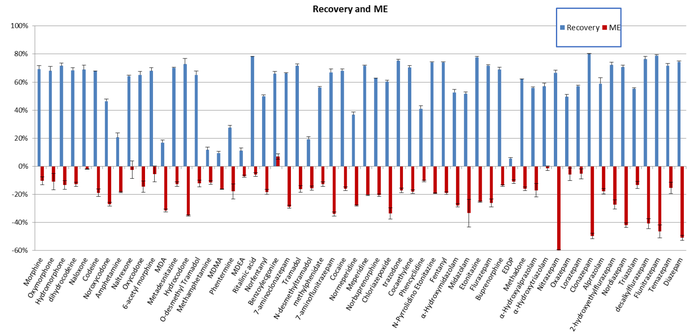


Figure 3. Recovery and matrix effects for all compounds in the multi-drug panel.

Recoveries and matrix effects (ME) for all analytes are shown in **Figure 3**. Recoveries ranged from 6-79% with 49/58 > 40%. All %RSDs were under 17%. Amine stimulants and EDDP had lower recoveries than other analytes, but they were consistent and enabled accurate quantification even at the lowest concentrations. Matrix effects ranged up to –59% with only 6 compounds exceeding 40% ion suppression. They were also consistent with all S.D. values <10%. Phentermine and metadesnitazine were subject to some endogenous interferences which interfered with their quantification. All other compounds were well controlled.

CONCLUSIONS

- A method for the extraction and quantitative analysis of multiple classes of drugs from hair was developed and optimized to balance extraction efficiency and the stability needs of all compounds.
- The resulting method readily passed quantitative validation criteria for all compounds (excepting phentermine and metadesnitazine)
- The sensitivity requirements of SoHT were met for all compounds
- All analytes were positively identified in external quality control samples, with good quantitative correlation
- An efficient and timely workflow enables extraction within a 3-4 hour timeframe, enabling same-day batch analysis

References: 1—Cooper et al. 2012. Forensic Sci. Int 281, 20-24.

Quantitative Analysis

Calibration curves ranged from 0.01-1.0 ng/mg for most drugs, with a few exceptions. Norfentanyl and 6-acetyl morphine ranged from 0.002-0.2 ng/mg and fentanyl ranged from 0.001-0.1 ng/mg. **Table 1** lists R^2 values for all analyte calibration curves. **Table 2** lists the intra-batch statistics for all the analytes in the panel. With the exception of phentermine and metadesnitazine, all compounds met validation criteria for accuracy and precision, both for intra-batch results (shown) and inter-batch results. **Table 2** also shows the cut-offs recommended by SoHT. All compounds satisfied the designated cut-off concentrations listed.

Table 2. Mean accuracy and precision for within batch QC results, together with lower limits of quantification (LLOQ) and SoHT cut-offs

	Within Batch Statistics							
	Low		Med		High		LLOQ	SoHT Cut-Off
	Mean	%CV	Mean	%CV	Mean	%CV		
Morphine	101.3	4.2	105.1	5.8	93.4	5.6	0.01	0.20
Oxymorphone	98.6	2.7	100.4	1.1	96.4	3.3	0.01	0.20
Hydromorphone	98.2	2.6	101.5	1.2	97.3	4.2	0.01	0.20
Dihydrocodeine	98.1	2.5	101.5	1.8	97.2	4.8	0.01	0.20
Naloxone	91.6	5.1	100.8	4.5	94.2	4.4	0.01	0.20
Codeine	92.0	8.5	98.4	0.7	94.5	4.3	0.01	0.20
Noroxycodone	94.7	7.7	99.5	5.8	94.0	4.3	0.01	0.20
Amphetamine	117.6	10.9	101.5	2.5	95.8	2.1	0.01	0.20
Naltrexone	102.5	4.7	98.8	3.3	91.6	4.5	0.01	0.20
Oxycodone	96.4	2.5	103.6	1.9	97.4	3.6	0.01	0.20
6-acetyl morphine	93.3	5.4	103.1	5.6	94.8	2.4	0.002	0.20
MDA	99.2	10.0	102.7	3.3	96.4	5.3	0.03	0.20
Metadesnitazine	95.9	4.1	97.6	2.3	86.9	3.4	—	—
Hydrocodone	98.2	4.1	103.5	1.6	94.5	3.6	0.01	0.20
O-desmethyl Tramadol	96.8	4.1	103.1	0.8	96.2	3.5	0.01	0.20
Methamphetamine	96.5	3.0	103.7	3.5	94.6	5.7	0.01	0.20
MDMA	96.2	6.1	102.0	5.1	97.8	6.9	0.01	0.20
Phentermine	ND	ND	44.0	85.5	92.3	7.2	—	0.20
MDEA	96.7	8.0	101.8	3.8	97.8	4.6	0.01	0.20
Ritalinic acid	82.7	9.0	94.7	7.1	94.3	5.1	0.01	n/a
Norfentanyl	91.5	4.3	101.4	2.8	105.8	5.1	0.002	0.20
Benzoylecgonine	100.5	3.2	100.9	1.5	94.5	2.8	0.01	0.05
7-aminoclonazepam	100.8	3.0	97.5	2.8	92.0	2.7	0.01	0.05
Tramadol	97.8	1.5	103.2	2.4	98.3	2.6	0.01	0.20
N-desmethyltramadol	96.5	5.5	105.2	3.4	96.0	3.0	0.01	0.20
Methylphenidate	99.3	1.7	101.0	1.6	95.5	2.7	0.01	—
7-aminoflunitrazepam	96.4	4.2	100.9	1.8	95.3	3.1	0.01	0.05
Cocaine	99.7	3.2	101.3	2.3	96.0	4.2	0.01	0.50
Normeperidine	97.2	4.1	102.4	1.6	96.7	3.2	0.01	0.01

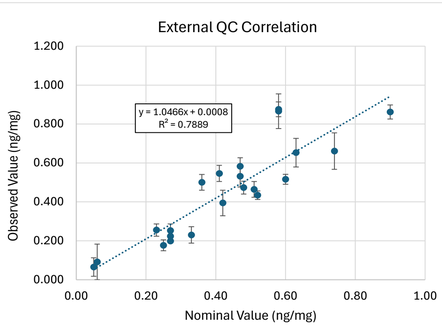
	Within Batch Statistics							
	Low		Med		High		LLOQ	SoHT Cut-Off
	Mean	%CV	Mean	%CV	Mean	%CV		
Meperidine	97.4	2.7	101.7	1.1	95.2	3.9	0.01	0.20
Norbuprenorphine	102.8	7.7	97.3	4.3	97.6	3.1	0.01	0.01
Chloriazepoxide	99.9	4.1	113.9	0.6	102.3	2.4	0.01	0.05
Trazodone	100.2	2.5	105.2	2.2	99.3	3.6	0.01	0.20
Cocaethylene	99.0	2.3	101.4	1.9	96.5	3.3	0.01	0.05
Phencyclidine	98.0	2.1	101.5	1.0	95.7	3.7	0.01	--
N-Pyrrolidino Etonitazine	98.6	3.7	100.1	2.9	90.5	3.2	0.01	--
Fentanyl	99.5	2.8	101.5	2.3	94.2	2.6	0.001	0.20
α-Hydroxymidazolam	108.7	7.9	105.8	3.2	93.6	3.3	0.03	0.05
Midazolam	94.6	4.3	102.0	1.7	96.6	1.9	0.01	0.05
Etonitazine	96.4	5.8	100.3	4.1	91.8	4.2	0.01	--
Flurazepam	98.6	3.6	103.5	2.5	99.8	2.1	0.01	0.05
Buprenorphine	96.6	6.5	97.4	4.8	93.4	4.3	0.01	0.01
EDDP	100.7	4.0	102.2	1.9	96.5	3.5	0.01	0.05
Methadone	99.3	3.9	103.7	2.1	97.3	2.9	0.01	0.20
α-Hydroxyalprazolam	92.9	9.1	101.7	2.6	97.1	2.1	0.01	0.05
α-Hydroxytriazolam	94.8	9.9	101.8	2.2	99.3	3.1	0.01	0.05
Nitrazepam	96.5	4.9	101.3	2.1	100.2	3.8	0.01	0.05
Oxazepam	103.7	11.2	107.6	6.0	94.1	5.0	0.02	0.05
Lorazepam	97.8	10.1	103.6	2.1	95.9	4.9	0.02	0.05
Clonazepam	104.6	5.4	106.0	4.4	93.3	3.3	0.03	0.05
Alprazolam	97.8	5.9	100.3	1.2	95.7	3.5	0.01	0.05
2-hydroxyethylflurazepam	101.8	8.5	102.6	7.3	96.7	4.0	0.01	0.05
Nordiazepam	98.7	2.7	101.6	2.8	96.5	4.0	0.01	0.05
Triazolam	102.5	3.4	103.0	3.9	94.1	2.2	0.01	0.05
Desalkylflurazepam	94.7	2.5	103.0	3.0	96.6	2.8	0.01	0.05
Flunitrazepam	93.9	1.7	101.6	2.1	95.2	3.1	0.01	0.05
Temazepam	96.0	4.0	101.6	2.3	97.2	0.9	0.01	0.05
Diazepam	96.0	2.1	98.5	2.3	96.2	2.6	0.01	0.05

External Assessment

Table 3. Observed mean values (*N*=5), nominal concentrations and reference ranges of external control samples. All included compounds were positively identified. Fentanyl and 6-acetyl morphine had values beyond the range of this assay. For the remaining compounds, 18/22 (82%) were within the control limits assigned by the manufacturer and all had %CVs <10%.

	EQC Result					
	Mean (ng/mg)	%CV	Nominal Conc.	Lower Limit	Upper Limit	Acceptable
Morphine	0.473	3.31	0.48	0.310	0.650	y
Dihydrocodeine	0.583	4.35	0.47	0.310	0.630	y
Codeine	0.253	3.41	0.27	0.180	0.360	y
Amphetamine	0.464	4.07	0.51	0.330	0.690	y
Oxycodone	0.532	5.51	0.47	0.300	0.630	y
6-acetyl morphine	0.841	2.68	0.57	0.370	0.770	n*
MDA	0.394	6.58	0.42	0.270	0.570	y
Methamphetamine	0.516	2.57	0.60	0.390	0.810	y
MDMA	0.661	9.37	0.74	0.480	1.000	y
MDEA	0.653	7.35	0.63	0.410	0.850	y
Benzoylecgonine	0.500	4.07	0.36	0.230	0.490	n
Tramadol	0.434	2.18	0.52	0.340	0.700	y
Cocaine	0.862	3.61	0.90	0.590	1.220	y
Norbuprenorphine	0.091	9.17	0.06	0.039	0.081	n
Cocaethylene	0.876	3.80	0.58	0.380	0.780	n
Fentanyl	0.224	3.17	0.14	0.090	0.190	n*
Buprenorphine	0.065	4.78	0.05	0.033	0.068	y
EDDP	0.546	4.17	0.41	0.270	0.550	y
Methadone	0.866	8.91	0.58	0.380	0.780	n
Lorazepam	0.198	3.18	0.27	0.180	0.360	y
Alprazolam	0.255	4.23	0.23	0.150	0.310	y
Nordiazepam	0.230	2.83	0.33	0.210	0.450	y
Temazepam	0.177	3.27	0.25	0.160	0.340	y
Diazepam	0.224	1.89	0.27	0.180	0.360	y

Figure 4. Correlation between the nominal values and our observed values. Each point represents a different analyte, the overall slope of 1.05 indicates excellent overall agreement with the assigned values.



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